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Title: Confirmation of Florfenicol Residues by GC/MS		
Revision: 01	Replaces: CLG-FLOR2.00	Effective: 02/08/2005

Contents

A. INTRODUCTION

1. Theory

Residues of florfenicol and its metabolites in bovine liver homogenate are converted to florfenicol amine (FA) salts by acid catalyzed hydrolysis. Dichloromethane is then added with mixing to extract neutral lipids. Following centrifugation, the aqueous layer is removed and made alkaline with NaOH to convert FA to its free base. FA is then extracted into ethyl acetate, and the extracts are evaporated to dryness. A cyclic boronate FA derivative is then prepared and analyzed by capillary gas chromatography/selective ion monitoring-mass spectrometry (GC/SIM-MS).

2. Scope

This method has been demonstrated to confirm the presence of florfenicol residues (as florfenicol amine) in bovine liver tissues at concentrations ≥ 1.9 ppm.

B. EQUIPMENT

Note: Equivalent equipment may be substituted unless otherwise specified.

1. Apparatus

a. Volumetric Flasks, glass - Kimax, 10, 100 and 500 mL, type TC, Fisher Scientific Co.

b. Glass Graduated Cylinders - 100, 250, 500 and 1000 mL, Type TC 20 °C, Fisher Scientific Co.

c. Analytical Balance - Leco-250, Leco Corp.

d. Test Tubes - Pyrex 16 x 125 mm borosilicate screw capped (caps are Teflon lined), Cat # 60827-533, VWR.

e. Pipettors - Rainin EDP variable volume micropipettes, 5 - 100 µL and 500 - 5000 µL, with Rainin pipette tips, Rainin Instruments Inc.

f. Vortex Mixer - Type 16700 mixer, Barnstead International.

g. Rotary Mixer - Glas-Col variable speed rotary mixer, Model 099A, Glas-Col Apparatus Co.

h. Pasteur Pipettes - disposable glass, 5.75 inch.

i. Centrifuge - Sorvall T 6000, Dupont.

j. Test Tubes - Pyrex 13 x 100 mm, screw capped tubes, Cat #60826-370, VWR. Order teflon lined caps separately Cat #60827-227, VWR.

k. Shaking Water Bath capable of maintaining 95 °C to 100 °C, Precision Scientific.

l. Beakers - 30 and 100 mL, Pyrex.

m. pH Paper - pHydrion Vivid 1-11, Microessential Laboratory.

n. Sample Concentrator - Meyer N-Evap, Organomation.

o. Food Processor - Robot Coupe Model RSI6Y-1.

2. Instrumentation

a. Gas Chromatograph/Mass Spectrometer - Agilent Model 5973 Network Mass Selective Detector, Model 6890 GC, with 7683 Series Injector and 7683 Series Autosampler, Agilent Technologies.

b. Fused Silica Capillary Column - J&W DB-17 liquid phase, 0.5µm film thickness, 15 m x 0.25 mm id., Agilent Technologies.

c. Injection port liner - deactivated, tapered end, packed with glass wool. Hewlett Packard part # 5062-3587, Agilent Technologies.

d. Auto Liquid Sampler Vials - 2 mL Screw cap vials with septa, Cat # 5182-0866, using conical inserts with polymer feet, Cat #5182-0549, Agilent Technologies.

C. REAGENTS AND SOLUTIONS

Note: Equivalent reagents and solutions may be substituted unless otherwise specified.

1. Reagents

a. Hydrochloric acid (HCl) - 36.5 - 38.0% - Cat. # JT9535-3, VWR.

b. Water - Deionized and charcoal filtered, prepared using Water Pro Plus Polishing station, Labconco.

c. Sodium hydroxide (NaOH) pellets - Sigma Cat. # S-5881, Sigma-Aldrich.

d. Methanol (MeOH), HPLC grade - Cat. # JT9093-3, VWR,

e. n-Butaneboronic acid - Cat. # 16,324-4, Sigma-Aldrich.

f. N,N-Dimethylformamide (DMF) - Cat. # MK492904, VWR.

g. Ethyl acetate (EtOAc) - Cat. # JT9282-3, VWR.

h. Dichloromethane (DCM) - Cat. # JT9315-2, VWR.

i. Nitrogen - 99.998% purity.

j. Helium - 99.9999% purity.

k. Perfluorotributylamine (PFTBA) - Cat. # 182014, SCM Chemicals.

2. Solutions

   a. 6N HCl:

      Add 200 mL deionized water to a screw capped glass bottle. Slowly add 200 mL of concentrated HCl. Mix thoroughly. Store tightly capped at room temperature.

   b. Saturated NaOH:

      Weigh 800 grams of NaOH pellets into a 1 L plastic container. Slowly add deionized water in approximately 100 mL portions, mixing after each addition until 1 liter of saturated NaOH solution is prepared. Wait 16 hours to ensure that visible NaOH remains undissolved. Store tightly capped at room temperature.

   c. Derivatizing Reagent:

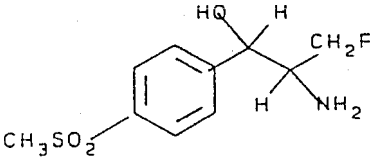
      Transfer 25 mg of n-butaneboronic acid to a 5 mL volumetric flask. Dilute to volume with N,N-dimethyl-formamide and mix thoroughly. Protect from light. Prepare fresh each day analysis is performed.

D. STANDARDS

1. Analytical Standard

   a. Name: Florfenicol Amine (FA) (D-(threo)-1-(p-methylsulfonylphenyl)-2-amino-3-fluoro-1-propanol)

   b. Chemical Structure:



   c. Molecular weight: 247.08

   d. Supplier: Schering-Plough (SCH 40458)

   e. Purity: ≥ 98%

   f. The reference standard will be supplied with a certificate of analysis indicating the exact purity for that batch, typically ≥ 98%.

   g. Storage: Store at room temperature (approximately 15 - 30 °C).

2. Standard Solutions

   a. FA Stock Standard (0.10 mg/mL):

      Weigh amount of FA equivalent to 50 ± 1 mg when corrected for purity, recorded to at least 3 significant figures, into a suitable vessel. Quantitatively transfer to a

500 mL volumetric flask, dilute to volume with MeOH, and mix well.

Transfer solution to a screw capped amber glass bottle and store refrigerated (approximately 4 -10 °C). Solution is stable for 6 months.

E. SAMPLE PREPARATION

1. Preparation of Liver Sample

   a. Prepare entire sample (or at least 450 g if sample weight exceeds 450 g). If sample is frozen, allow to thaw. Trim sample of extraneous connective tissue. Cut entire sample into thin slices or small cubes. If only a sub-sample is to be prepared, assure that the portions selected are representative of the entire sample.

   b. Using a food processor, prepare a homogeneous sample mixture by thoroughly blending liver tissue. Store frozen until needed for testing. Take care to avoid any unnecessary subsequent thaw/freeze cycles.

2. Preparation of Test Sample

   a. Allow sample to thaw. If frozen sample is homogeneous, only a portion need be thawed for testing.

   b. Stir thawed sample portion, if necessary, to re-mix.

F. ANALYTICAL PROCEDURE

1. Hydrolysis and Cleanup

   a. For each test sample, carefully weigh a 1 ± 0.1 g tissue aliquot into a 16 x 125 mm screw capped tube.

      Note: Prepare control samples at this time. Weigh two 1 ± 0.1 g portions of blank control homogenate (liver predetermined to contain no interferences). Fortify one portion with florfenicol standard stock solution. Fortification level of this control should approximately match that expected in the sample to be confirmed. For example, a 3.7 ppm control requires addition of 37 µL of 0.10 mg/mL florfenicol standard.

   b. Add 5 mL of 6N HCl to each tube in the sample set.

   c. Vortex mix for 5 seconds.

   d. Place tube in a shaking water bath set to at least 95 °C, for at least 2 hours. Alternatively, a boiling water bath may be used. Briefly remove tube from the water bath and mix on a vortex mixer every 10 to 15 minutes during hydrolysis. It is important to mix or agitate the sample during hydrolysis to ensure complete digestion.

e. After 2 hours, remove tube from the bath and inspect its contents. The solution should be dark brown to black with only charred black flocculent material remaining. If pieces of undigested (brown or dark gray in color) liver remain, continue heating and mixing until tissue is completely digested.

f. Remove tube from the water bath and allow to cool to room temperature.

Stopping point. The procedure maybe stopped at this point for a short period of time, approximately 2 - 3 hours at room temperature.

g. Add 5 ml of dichloromethane. This step should be performed in a well ventilated hood.

h. Cap tube tightly and rotary mix at 20 ± 2 rpm for 15 minutes.

i. Centrifuge at approximately 1860 x g (~3300 rpm) for 10 minutes.

j. Using a disposable borosilicate glass pasteur pipette, carefully transfer only the upper aqueous layer into a new 16 x 125 screw capped test tube, leaving the black tarry interface behind. Do not disturb or transfer the black tarry interface. Discard tube containing dichloromethane residues.

k. While vortexing tube containing acidic digest, slowly add 3.0 ml saturated NaOH in small increments, then vortex tube for an additional 10 seconds.

Caution! Highly exothermic reaction! Wear eye and hand protection.

l. Allow tube to cool to room temperature.

m. Add 4.5 mL of ethyl acetate to tube, cap tightly, and rotary mix at 20 ± 2 rpm for 15 minutes.

n. Centrifuge tube at approximately 1860 x g (~3300 rpm) for 10 minutes.

o. Using a disposable pasteur pipette, carefully transfer only the upper organic layer into a 13 x 100 mm borosilicate culture tube.

p. Place tube in sample concentrator (55 - 65 °C), and evaporate solution to dryness under a stream of nitrogen.

q. Repeat extraction and concentration (steps m - p) twice more, using the same culture tube to collect all extracts.

Stopping Point. Samples can be stored in a freezer (≤ -10 °C) for 1 to 5 days.

r. Prepare external standards for derivatization at this time by pipetting suitable aliquots of florfenicol amine stock standard into clean 13 x 100 mm borosilicate culture tubes adding 4.5 mL of ethyl acetate, and evaporating to dryness as described in step E.2.p above. Concentration of standard should be approximately equal to that expected to be confirmed in the test sample extract.

2. Derivatization

   a. Add 100 µL of Derivatization Reagent to culture tube.

   b. Vortex mix tube briefly (5 - 10 seconds). Allow tube to remain at room temperature for 5 minutes.

   c. Transfer derivatized extract to an autosampler vial insert and immediately seal vial. Transfer and seal one extract at a time.

      Note: Derivatized samples should be analyzed on the day of derivatization. If this is not possible, store derivatized extracts at room temperature and analyze within 24 hours.

3. GC/MS Analysis

   a. Set Instrumental parameters as specified below.

      Note: Parameters may be adjusted, if necessary, to optimize system resolution and sensitivity. System should be tuned and demonstrated to be in good working condition before analyses are attempted.

      Gas Chromatograph Conditions

Carrier gas:	99.9999 % Helium.
Secondary regulator pressure:	350 kPa.
Inlet Pressure:	50 kPa.
Carrier gas linear velocity:	48 cm/second.
Injection volume:	1 µL.
Injection mode:	Split. Split ratio: 12:1.
Injection port liner:	Place tapered end toward column.
Injection port temperature:	300 °C.
Column oven temp. program:	125 °C (1 minute hold) to 280 °C at 25 °C/minute. Hold at 280 °C for 5 minutes.
FA boronate retention time:	Approximately 7 - 8 minutes.

Mass Spectrometer Settings	
Transfer line temperature:	280 °C.
Electron Multiplier Voltage:	200 volts above autotune.
Electron Energy:	68 eV.
Calibration Standard:	PFTBA.
Emission Current:	220 µA.
Mass Spec on:	4.5 minutes (filament on).
SIM dwell times	50 milliseconds per ion.
FA Boronate Ions monitored: (m/z ± 0.5 AMU)	117 $[M\text{-}CH_3SO_2]$ ++ 130 $[C_4H_9BNHCHCH_2F]H$ + 280 $[M\text{-}CH_2F]$ + (Base Peak)

b. Analyze the following in one contiguous run to assure consistent instrument response: FA standard(s), control liver, control liver fortified with FA, derivatized sample extracts, and at least one FA standard at the end of the injection sequence. Replicate injections of standards and test samples may be made and results averaged to reduce errors due to random response fluctuation, if necessary.

c. Example Chromatograms (See Appendix, Section K.2)

G. CONFIRMATION

1. Analyze MS data for each injection:
   a. Generate individual ion chromatograms for ions 117, 130, and 280.
   b. Determine retention times and peak areas or peak heights for those ions.
   c. Calculate the following m/z ratios based on peak area or peak height measurements: 117/280, 130/280.

2. Verify that instrument response to external standards shows adequate sensitivity and is acceptably constant over the course of the CG/MS run. If these criteria are not met, confirmation should not be attempted.

3. A test sample will be confirmed if:
   a. The retention time of the florfenicol amine boronate peak in the m/z 280 ion chromatogram is within 2% of that observed for an appropriate external standard(s) included in the analysis set.

b. Ion abundance ratios 117/280 and 130/280 calculated for the test sample agree with those of an appropriate external standard(s) included in the analysis set to within ± 10%. Note: The tolerance is absolute. For example, to meet the tolerance when compared against a standard showing a 70% ratio, a sample would have to show a ratio ratio between 60% and 80%.

H. SAFETY INFORMATION AND PRECAUTIONS

1. Required Protective Equipment - Safety glasses, lab coat, protective gloves.

2. Hazards

Method Step	*Hazard*	*Recommended Safe Procedures*
B.1. Concentrated acids and bases HCl, NaOH	Corrosive. Contact with liquids can result in burns and severe skin, eye, and respiratory irritation (HCl).	Prepare solutions using these reagents with care in a well-ventilated area such as a fume hood. Wear protective eyewear, gloves, and clothing when handling.
B.1 Organic solvents: DCM, EtOAc	Flammable, vapors are corrosive to the skin, eyes, and respiratory system. DCM is a possible carcinogen.	Use only in an efficient fume hood, away from any electrical or heating devices
E.2.k. pH adjustment	Addition of strong base to strong acid can produce violent exothermic reaction, with possibility of eruption of acid or basic liquid.	Wear gloves and protective eyewear, gloves, and clothing. Add NaOH slowly to acid and mix thoroughly

3. Disposal Procedures

Method Step	*Hazard*	*Recommended Safe Procedures*
DCM wastes	See above	Collect and store in approved collection container until disposed of by a contractor or an in house specialist.
Basic/Acid wastes	See above	Neutralize solutions to meet local, state, and federal guidelines before disposal via sanitary sewer.

I. QUALITY ASSURANCE PLAN

1. Performance Standard

Analyte	*Analytical Range*	*False Negative Rate*	*False Positive Rate (0 conc.)*
FA	≥1.9 ppm	0	0

2. Critical Control Points and Specifications

Step	*Record*	*Acceptable Control*
E.2.j	Phase transfer	No organic phase or interface
E.2.p	Phase transfer	Transfer no aqueous phase
E.2.r	Concentrate to dryness	Allow no solvent to remain

3. Readiness To Perform (FSIS Training Plan)

   a. Familiarization

      i. Phase I: Standards- Prepare external standards equivalent to sample concentrations of 1.9 ppm florfenicol amine and analyze over 3 different days to demonstrate instrument sensitivity.

      ii. Phase II: Fortified samples - Analyze a blank liver and duplicate recoveries fortified at 1.9 ppm on each of 3 different days (9 analyses).

      Note: Phase I and Phase II may be performed concurrently.

      iii. Phase III: Check samples for analyst accreditation.

         (a) Analyze 6 samples consisting of one blank and 5 recoveries at concentrations between 1.9 and 3.7 ppm, all blind to the analyst.

         (b) Report analytical findings to supervisor.

         (c) Certification from Quality Assurance Manager (QAM) is required to commence official analysis.

   b. Acceptability criteria.

      Refer to section I.1 above.

4. Intralaboratory Check Samples

   a. System, minimum contents.

      i. Frequency: One per week per analyst on weeks when analyses are conducted.

ii. Records are to be maintained for review

b. Acceptability criteria.

Refer to section J.1 above.

If unacceptable values are obtained, then:

i. Stop all official analyses by that analyst.

ii. Take corrective action.

5. Sample Acceptability and Stability

a. Matrix: Bovine liver

b. Minimum Sample Size: 250 g.

c. Condition Upon Receipt: Frozen or with ice crystals

d. Sample storage:

i. Time: One year

ii. Condition: Frozen at ≤ -10 °C

6. Each sample set must contain:

a. Blank Control

b. Fortified control

c. Samples to be confirmed.

7. Sensitivity

Minimum proficiency level (MPL): 1.9 ppm FA.

J. WORKSHEET

Reserved

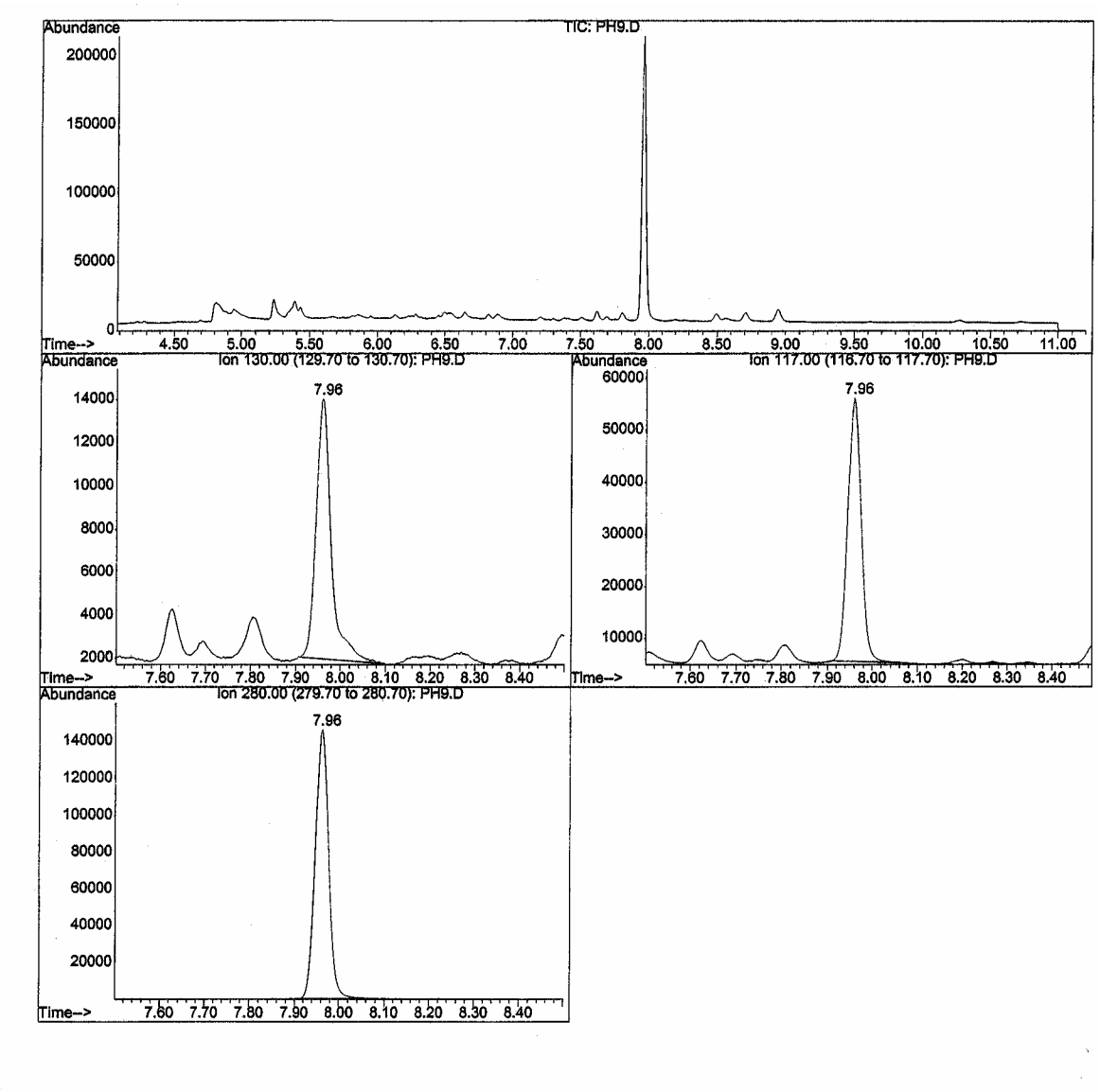
K. APPENDIX

1. Method Reference:

"SCH 25298 (Florfenicol): Gas Chromatography/Mass Spectrometric Method For the Determination of Florfenicol Amine in the Target Tissue (Liver) From Cattle Treated With Florfenicol (Revision Date November 3, 1995)". Alice M Bova, Drug Safety and Metabolism - Animal Health, Safety Evaluation Center, Schering-Plough Research Institute, Lafayette, New Jersey.

2. TIC and Ion Chromatograms

Example of TIC and Monitored Ion Chromatograms from Recovery Fortified at 3.9 ppm



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